CUKUROVA UNIVERSITY

INSTITUTE OF NATURAL AND APPLIED SCIENCES

MSc THESIS

Investigation of LHX4 Gene Polymorphism in Awassi and Cukurova Meat Sheep

HADIL FOUAD MIDHAT AL BAYATI

Department of Animal Science

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This Master's Thesis was evaluated by the following Jury Members on 25/06/2024 and was approved by unanimity / ~~majority~~ of votes.

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ABSTRACT

This study aimed to investigate the effect of LHX4 gene polymorphisms on growth traits such as height at the wither (cm), height at the rump (cm), chest depth (cm), body length (cm), chest width (cm), rump width (cm), chest girth (cm), and body weight (kg) in Awassi and Çukurova Meat Sheep (ÇET) breeds. Blood samples were collected from a total of 99 animals, 48 of which were Awassi and 51 of which were ÇET breeds, and genomic DNA was isolated from these blood samples at Çukurova University Faculty of Agriculture Small Ruminant Breeding Farm. The LHX4 gene region was screened by PCR using two different primer sets (LHX4-P2 and LHX4-P3). D (282 bp) and I (294 bp) alleles were determined in the amplicons of both primers, and II (insertion/insertion) and DD (deletion/deletion) genotype frequencies were calculated. At the LHX4-P2 locus, DD and II genotype frequencies were calculated as 42% and 58% for the Awassi breed, respectively, whereas genotype frequencies for ÇET breed were calculated as 40% and 60%, respectively. At the LHX4-P3 locus, DD and II genotype frequencies were calculated as 62% and 38% for the Awassi breed and 70% and 30% for ÇET breed, respectively. Individuals with heterozygous genotypes were not identified in the study. No significant relationship was found between the DD and II genotypes of the LHX4-P2 and LHX4-P3 loci and growth traits in either breed.

Keywords: Sheep, Awassi, ÇET, LHX4 gene, growth traits

ÇUKUROVA ÜNİVERSİTESİ
FEN BİLİMLERİ ENSTİTÜSÜ

YÜKSEK LİSANS TEZİ

İvesi ve Çukurova Et Koyunlarında LHX4 Gen Polimorfizminin Araştırılması

HADIL FOUAD MIDHAT AL BAYATI

Danışman: Prof. Dr. Devrim ÖZCAN

Zootekni Anabilim Dalı

ÖZ

Bu araştırma, İvesi ve Çukurova Et Koyun (ÇET) ırklarında LHX4 gen polimorfizmlerinin cidago yüksekliği (cm), sağrı yüksekliği (cm), göğüs derinliği (cm), vücut uzunluğu (cm), göğüs genişliği (cm), sağrı genişliği (cm), göğüs çevresi (cm) ve canlı ağırlık (kg) gibi büyüme özellikleri üzerine olan etkisini araştırmak amacı ile yapılmıştır. Çukurova Üniversitesi Ziraat Fakültesi Küçükbaş Hayvan Yetiştirme Çiftliği'nde 48'i İvesi ve 51'i ÇET ırkı olmak üzere toplam 99 hayvandan kan örnekleri alınmış ve bu kan örneklerinden genomik DNA'lar izole edilmiştir. LHX4 gen bölgesi iki ayrı primer seti (LHX4-P2 ve LHX4-P3) kullanılarak PCR yöntemi ile taranmıştır. Her iki primere ait amplikonlarda D (282 bp) ve I (294 bp) allelleri belirlenmiş ve buna göre II (insörsiyon/insörsiyon) ve DD (delesyon/delesyon) genotip frekansları hesaplanmıştır. LHX4-P2 lokusunda İvesi ırkı için DD ve II genotip frekansları %42 ve %58 olarak hesaplanırken, ÇET ırkı için genotip frekansları aynı sırayla %40 ve %60 olarak hesaplanmıştır. LHX4-P3 lokusunda ise İvesi ırkı için DD ve II genotip frekansları %62 ve %38 olarak hesaplanırken, ÇET ırkı için genotip frekansları yine aynı sırayla %70 ve %30 olarak hesaplanmıştır. Çalışmada heterozigot genotipe ait bireyler bulunamamıştır. Her iki ırkta da LHX4-P2 ve LHX4-P3 lokusları için DD ve II genotipleri ile büyüme özellikleri arasında önemli bir ilişki bulunmamıştır.

Anahtar Kelimeler: Koyun, İvesi, ÇET, LHX4 geni, büyüme özellikleri

GENİŞLETİLMİŞ ÖZET

Hayvan yetiştiriciliğinin en önemli temel hedefi çiftlik hayvanlarının bireysel olarak genetik yapılarını ve gelecek nesil popülasyonunun genetik bileşimini geliştirmektir. Çiftlik hayvanları içerisinde binlerce yıldır önemli bir konuma sahip olan koyunlar, hayvan genetiği ve biyoteknoloji alanlarında kat edilen ilerlemeler sayesinde önemli ölçüde gelişim göstermiştir. Genel olarak ıslah programları, genetik çeşitliliği artırarak ve istenilen özelliklere sahip bireylerin seçimini optimize ederek, koyun popülasyonlarının verimliliğini ve adaptasyon yeteneklerini iyileştirmeyi amaçlamaktadır. Özellikle et, süt ve yapağı üretimi gibi ekonomik açıdan önemli özellikler ile hastalıklara ve zorlu çevre koşullarına karşı dirençlilik, ıslah hedefleri arasında öncelikli yer tutar. Modern ıslah yöntemleri arasında yer alan yapay tohumlama, embriyo transferi ve genomik seleksiyon gibi teknikler genetik ilerlemenin hızlandırılmasına ve daha verimli koyun sürülerinin oluşturulmasına olanak tanımaktadır. Türkiye'de de, yerel koyun ırklarının genetik potansiyelini ortaya çıkarmak ve ülke koşullarına uygun, yüksek verimli yeni ırklar geliştirmek amacıyla yapılan ıslah çalışmaları hem yerli koyun popülasyonlarının korunmasına hem de ülke ekonomisine katkı sağlamaktadır.

Genetik seleksiyon, hayvanlarda genetik çeşitliliği artırarak ve hedeflenen fenotipik özellikleri optimize ederek, verimliliklerini ve adaptasyon yeteneklerini geliştiren bir yöntemdir. Bu yöntemin temeli popülasyonları oluşturan bireylerde genetik potansiyelin analizi, bu analiz sonucunda istenilen özellikleri taşıyan bireylerin belirlenmesi ve bu bireylerin genetik materyalinin gelecek nesillere aktarılmasına dayanır. Klasik seleksiyon yöntemlerinin aksine, günümüzde uygulanan modern genetik seleksiyon yöntemleri, hayvanların fenotipik verilerine ilave olarak, genetik markörlerin kullanılmasıyla daha da hassas ve etkili bir hale gelmiştir. Bu süreçte popülasyon bir bütün halinde değil, bireylerin genetik yapıları geniş bir yelpazede analiz edilir. Böylece, hayvanların et, süt ve yapağı gibi ekonomik öneme sahip özellikleri iyileştirilebildiği gibi, aynı zamanda hastalıklara karşı dirençlilikleri de artırılmaktadır. Sonuç olarak, genetik seleksiyon, tarım ve hayvancılık sektörlerinde verimlilik artışı ve sürdürülebilirlik sağlamak adına kritik bir araçtır ve bu alanlardaki bilimsel araştırmalar, uygulamaların etkinliğini artırmak için sürekli olarak yenilikler sunmaktadır.

LIM1 ve LIM2'yi içeren LIM (Lin11/lsl1/Mec3) domainleri, sistein bakımından zengin iki adet çinko bağlayıcı domaine sahiptir ve amino terminali korunmuş bir DNA bağlayıcı homeodomain'e (HD) yerleştirilmiştir. LIM-HD domainleri sadece LIM domainlerine sahip olmakla kalmayıp aynı zamanda bir homeodomain'e de sahiptir. Bu nedenle bu ailenin üyelerinin hücre tipi spesifikasyonu ve farklılaşmasının çeşitli aşamalarını düzenleyen transkripsiyon faktörleri olarak işlev gördüğü gösterilmiştir. Bugüne kadar, memelilerde aralarında ISL1, ISL2, LHX2, LHX3 ve LHX4 gibi gelişimsel düzenleyici proteinleri kodlayan genlerin de olduğu en az 12 LIM-HD belirlenmiştir.

LIM homeobox transkripsiyon faktörü 4 (LHX4) geni fiziksel olarak koyunlarda 12. kromozom üzerinde bulunup, 7 adet ekzondan meydana gelmektedir (NCBI gen ID: 101122876). Fakat ekzon sayıları ve bulunduğu kromozom memelilerde farklılık göstermektedir. Örneğin insanlarda bu gen 6 adet ekzon ve 5 adet introndan meydana gelmiş olup, 1. kromozom üzerinde yer alırken, sığırlarda ve keçilerde yine 6 adet ekzondan meydana gelmiş olup, 16. kromozom üzerinde yer almaktadır.

LHX4, hipofiz bezi ve sinir sistemi gelişiminde önemli roller oynayan LIM-HD transkripsiyon faktörlerinden biridir. Bu gen sırasıyla adrenokortikotropik hormon (ACTH), follikül uyarıcı hormon (FSH), luteinleştirici hormon (LH), tiroid uyarıcı hormon (TSH), büyüme hormonu (GH) ve prolaktin (PRL) hormonlarının dâhil olduğu beş farklılaşmış hormon salgılayan hücrenin gelişimini düzenleyebilir. Bu hormonlar insan ve çiftlik hayvanlarında büyüme ve metabolizmayı, üreme gelişimini ve işlevini, tiroid fizyolojisini, laktasyonu ve stres tepkisini düzenler. LHX4, ön hipofizin erken embriyonik gelişimi için çok önemli bir transkripsiyon faktörüdür ve LHX3 (LIM homeobox gen 3) ile etkileşime girer ve memelide PROP1 ve POU1F1'i düzenler. Çiftlik hayvanlarında, POU1F1, GH ve PRL süt performansı, büyüme, üreme, endokrin, bağışıklık ve hastalıkta çeşitli kritik düzenleyici roller oynamaktadır. PROP1, POU1F1, GH ve PRL'yi düzenleyen LHX4 geninin hayvansal üretimde CPHD (combined pituitary hormone deficiency, kombine hipofiz hormon eksikliği) ve üretim özellikleri ile ilişkili olabileceği de düşünülmektedir.

Protein kodlayan bir gen olan LHX4 geninin 1. intronunda meydana gelen 12 bç'lik indel mutasyonu sonucu oluşan genotipler keçilerde bir batında doğan yavru sayısı ile ilişkilendirilmiş ve DD ve ID genotipine sahip bireylerde II genotipine sahip bireylere göre bir batında doğan yavru sayısı daha yüksek bulunmuştur. Gende meydana gelen bu 12 bazlık delesyon 282 bç'lik D allelinin meydana gelmesini sağlamıştır. Delesyon bulunmayan allel ise (I) 294 bç'nden meydana gelmektedir. Dolayısıyla DD (delesyon/delesyon) genotipleri agaroz jel elektroforezde 282 bç'lik ve II (insörsiyon/insörsiyon) genotipleri 294 bç'lik tek bir bant üretirken, D/I (delesyon/insörsiyon) heterozigot genotipler 282 ve 294 bç'lik 2 adet bant üretmektedir.

İvesi koyunu Türkiye'de eti ve sütü için yetiştiriciliği yapılan ırklardan birisi olup bir takım hastalıklara ve parazitlere karşı dirençlidir. ÇET (Çukurova Et Koyunu) ise Çukurova Üniversitesi'nde, Güneydoğu Anadolu'nun iklim koşullarına uyum sağlayabilecek, İvesi ile Sakız koyunlarının karakteristik özelliklerini taşıyan yeni bir koyun ırkı geliştirmek amacıyla, Ile de France, Rambouillet, İvesi ve Sakız ırklarının kullanıldığı melezleme çalışmaları sonucunda meydana getirilmiş bir ırktır.

Bu çalışma Çukurova Üniversitesi Ziraat Fakültesi Araştırma Uygulama Çiftliği Küçükbaş Hayvancılık Ünitesinde bulunan İvesi ve Çukurova Et Koyunu (ÇET) ırklarında Lim Homeobox Transcription Factor 4 geni lokusunun iki ayrı primer (LHX4-P2 ve LHX4-P3) bakımından genotiplerinin belirlenmesi ve elde edilen genotiplerin cidago yüksekliği (cm), sağrı yüksekliği (cm), göğüs derinliği (cm), vücut uzunluğu (cm), göğüs genişliği (cm), sağrı genişliği (cm), göğüs çevresi

(cm) ve canlı ağırlık (kg) gibi büyüme özellikleri üzerine olan etkisini araştırmak amacı ile yapılmıştır. Çalışmada İvesi ırkından 48 ve ÇET ırkından 51 olmak üzere tamamı 8 yaşında olan toplam 99 hayvan rastgele seçilmiştir. Hayvanlardan kan örnekleri jugular vein (*Vena jugularis*)'den EDTA içeren tüplere alınmış ve soğuk zincirde laboratuvara taşınmıştır. Kan örnekleri kullanılıncaya kadar -20 °C'de muhafaza edilmiştir. Kan örneklerinden genomik DNA'lar tuzla çöktürme (salting-out) protokolü kullanılarak elde edilmiştir. Miktar ve saflık kontrolleri yapılan DNA örnekleri PCR çalışmalarında kullanılmıştır. PCR sonucunda elde edilen amplikonlar agaroz jelde 100 bç'lik markır DNA ile birlikte yürütülmüş ve sonrasında jel görüntüleri fotoğraflanmıştır. Jeldeki amplikonların yer aldığı bant pozisyonları dikkatli bir şekilde incelenerek delesyonlu ve insörsiyonlu alleller tespit edilmiştir. İvesi ve ÇET ırklarına ait LHX4-P2 ve LHX4-P3 lokuslarından elde edilen polimorfizmlerinin çeşitli büyüme özellikleri üzerindeki etkisini belirlemek için tek yönlü varyans analizi (One-Way ANOVA) kullanılmıştır. Analizler Minitab 19 yazılımı kullanılarak gerçekleştirilmiştir. POPGENE versiyon 1.32, allel ve genotip frekanslarının tahmini değerleri için Hardy-Weinberg testini ($P \leq 0.05$) hesaplamak için kullanılmıştır. Tek Yönlü ANOVA kullanılarak varyans analizinde $Yij=\mu+\alpha i+\epsilon ij$ istatistiksel modeli kullanılmıştır.

Her iki primere ait amplikonlarda D (282 bp) ve I (294 bp) allelleri belirlenmiş, buna göre II ve DD genotip frekansları hesaplanmıştır. LHX4-P2 lokusunda İvesi ırkı için DD ve II genotip frekansları %42 ve %58 olarak hesaplanırken, ÇET ırkı için genotip frekansları aynı sırayla %40 ve %60 olarak hesaplanmıştır. LHX4-P3 lokusunda ise İvesi ırkı için DD ve II genotip frekansları %62 ve %38 olarak hesaplanırken, ÇET ırkı için genotip frekansları yine aynı sırayla %70 ve %30 olarak hesaplanmıştır. Çalışmada her iki ırkta da heterozigot genotipe ait bireyler bulunamamıştır. Heterozigot genotipe sahip bireylerin olmaması nedeniyle DD genotip frekansları ile D allel frekansları, II genotip frekansları ile de I allel frekansları aynı hesaplanmıştır. Buna göre, LHX4-P2 lokusunda İvesi ırkı için D ve I allel frekansları %42 ve %58 olarak hesaplanırken, ÇET ırkı için allel frekansları aynı sırayla %40 ve %60 olarak hesaplanmıştır. LHX4-P3 lokusunda ise İvesi ırkı için D ve I allel frekansları %62 ve %38 olarak hesaplanırken, ÇET ırkı için allel frekansları yine aynı sırayla %70 ve %30 olarak hesaplanmıştır. Her iki ırkta da LHX4-P2 ve LHX4-P3 lokusları için DD ve II genotipleri ile cidago yüksekliği (cm), sağır yüksekliği (cm), göğüs derinliği (cm), vücut uzunluğu (cm), göğüs genişliği (cm), sağır genişliği (cm), göğüs çevresi (cm), vücut ağırlığı (kg) arasında önemli bir ilişki bulunmamıştır.

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Furthermore, I extend my heartfelt appreciation to my parents, siblings, and extended family for their steadfast support and patience throughout the duration of my research and the writing of this thesis. Your persistent prayers and encouragement have provided me with immense strength.

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SYMBOLS AND ABBREVIATIONS

A113G : A→G conversion at position 113 of LEP
BW : Body weight
ACTH : Adrenocorticotropic hormone
BS : Body size
bp : Base pair
BFTW : Back Fat Thickness Weight
BMPRIB : Bone Morphogenetic Protein Receptor IB
CG : Chest girth
CW : Carcass weight
CAST : Calpastatin
CPHD : Combined Pituitary Hormone Deficiency
CSN2 : Casein 2
CCNB2 : Cyclin B2
ÇET : Çukurova Meat Sheep
DNA : Deoxyribonucleic Acid
DGAT1 : Diacylglycerol Acyltransferase1
EDTA : Ethylenediaminetetraacetic acid
FAO : Food and Agriculture Organization of the United Nations
FAOSTAT : Food and Agriculture Organization Corporate Statistical Database
FAP : Fatty acid profiles
FR : Fat rate
FecL : Fecundity L gene
GH : Growth hormone
GH1 : Growth hormone 1
GT : Growth traits
GRM1 : Glutamate Metabotropic Receptor 1
gr : Gram
GDF9 : Growth Differentiation Factor 9
ILV : Infection to the Lentivirus
LALBA : Alpha-lactalbumin
LHX3 : LIM homeobox 3 gene
LHX4 : LIM homeobox 4 gene
LEP : Leptin
LIM : Lin11/lsl1/Mec3
LIM-HD : LIM-homeodomain

MSTN : Myostatin
MEF2B : Myocyte Enhancer Binding Factor 2 B
MBD5 : Methyl CpG Binding Domain Protein 5
M : Million
ml : Milliliter
µl : Microliter
MY : Milk yield
MQ : Meat quality
OD : Oocyte development
ORS : Ovulation rate and sterility
PCR : Polymerase Chain Reaction
PRLR : Prolactin Receptor
PALMD : Palmdelphin
POU1F1 : POU Class 1 Homeobox 1
Prl : Prolactin
PR : Protein rate
pmol : Pico mole
PCR-SSCP : PCR- Single Strand Conformation Polymorphism
PWG : Post-weaning gain
RPL7 : Ribosomal Protein L 7
RP : Reproductive performance
RFP145 : Ring Finger Protein 145
SLC58A3 : Solute Carrier Family 8 Member A3
SCFD : Subcutaneous carcass fat depth
SMC2 : Structural Maintenance of Chromosome 2
TRHDE : Thyrotropin-releasing hormone degrading enzyme
TP53 : Tumor protein 53
TBFW : Total body fat weight
TBE : Tris-Borate-EDTA
TMEM154 : Transmembrane protein 154
UCP1 : Uncoupling protein 1
UBR2 : Ubiquitin Protein Ligase E3 Component n-Recognin 2
WW : Weaning weight

1. INTRODUCTION

Domestic sheep *Ovis aries* are (cud-chewing) ruminants that are economically and culturally essential farm animals. Sheep are mostly raised for milk, meat, fur, and wool production on farms. These products are essential for humans (Montossi et al., 2013). The sheep regurgitates its meal and chews cud, allowing its four different stomach compartments to fully digest the grasses and other herbage it consumes. Compared to cattle, they graze plants closer to their roots. They reach maturity at about one year of age, and many reproduce at one and a half years. Most sheep births are single, but twins can occasionally occur. At around four or five months of age, the lambs stop suckling and begin to graze. However, 1520 sheep breeds were identified in 2018, with local sheep breeds making up 80% of all sheep breeds globally (Kawecka et al., 2022).

Sheep are considered to have been domesticated around 10,000 years ago (Zeder, 2008). Sheep and goats were the initial livestock species to be domesticated (Ryder, 1983). They are the second-known domesticated animal, after dogs (Ankarali, 1988). In the seventh millennium BCE, they might have also independently domesticated in Mehrgarh, South Asia (modern-day Pakistan) (Franke, 2016; Meadow, 1991).

Today's domestic sheep originates from Mouflon (*Ovis oriantalis, Ovis musimon*) (Figure 1.1.), Arkar (*Ovis vignei*), and Argali (*Ovis ammon*) wild sheep, which are known to be resistant to adverse environmental conditions and have high reproductive ability. It is accepted that the first domestication region of wild sheep was Central Asia. It is known that the domestication of sheep from their wild forms is based on the emotional approaches of people and religious reasons in addition to their productivity characteristics (Kaymakçı, 1990; Kaymakçı, 2006; Karakuş et al., 2015).

Figure 1.1. European mouflon. Photo Credit: © Patrik Michál from CIC Caprinae Atlas of the World, Damm and Franco, 2014.

Sheep have always played an essential role in the survival of human settlements. Even though the world has grown rapidly in the last few centuries, sheep have remained extremely important. China has more than 175 million sheep, accounting for 13.7% of the world's sheep population. Several other countries also have large sheep populations. Australia follows after China. Australia has about 75 million sheep living within its borders. And India has more than 54 million sheep living within its borders. Therefore, sheep may be found all over the world in enormous numbers (sheep population by country, 2024). The Sheep populations are available in (Table 1.2 and Figure 1.1.) based on FAOSTAT (2024).

Table 1.1. Sheep populations throughout the countries (FAOSTAT, 2024)

Country	**Sheep Population (M)**	**Global (%)**
China	173.1	13.7
India	68.1	5.39
Australia	63.5	5.03
Nigeria	47.7	3.78
Iran	46.6	3.69
Ethiopia	42.9	3.4
Türkiye	42.1	3.34
Sudan	40.9	3.24

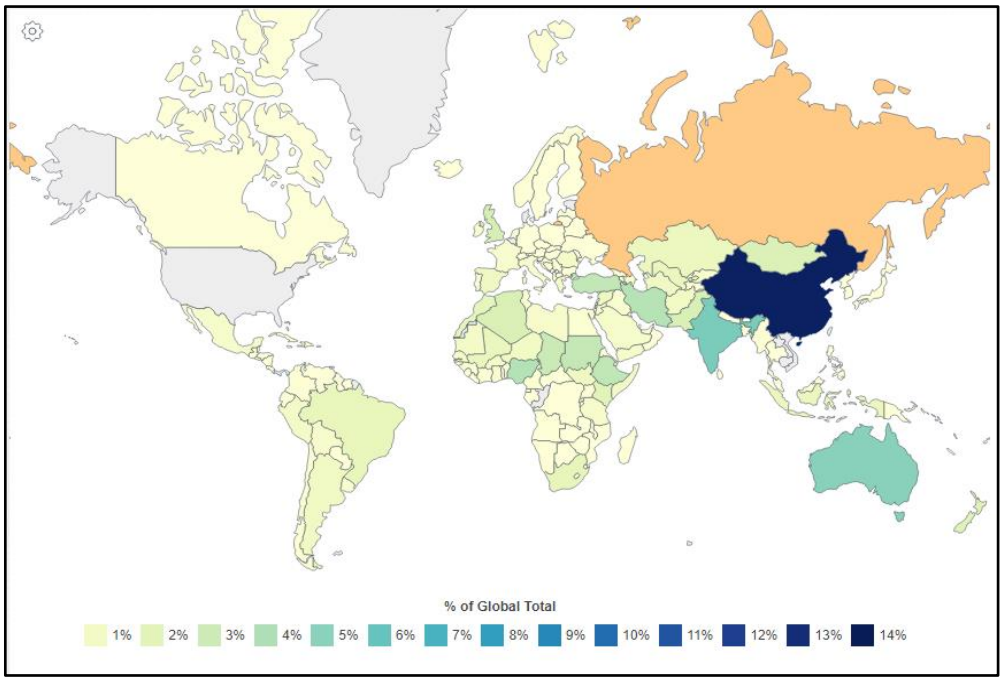

Figure 1.2. Sheep population in the world by country in 2024 (https://worldpopulationreview.com/country-rankings/sheep-population-by-country)

According to TÜİK (2022-2023), statistics in Türkiye, the number of big cattle decreased by 2.6% to 16 million, 583 thousand, belonging to the category of small-ruminant animals, the sheep population decreased by 5.9% to 42 million, 60,000 from 2022, and the number of goats decreased by 11.0% to 10 million, 330,000. The numbers of ruminants in 2022 and 2023 are given in Table 1.2. and Figure 1.3.

Table 1.2. Number of ruminants in 2022 and 2023 (TÜİK, 2023)

Genus	Years		Change (%)
	2022	**2023**	
Large ruminants	**17.023.791**	**16.583.005**	**-2,6**
Cattle	16.851.956	16.421.256	-2,6
Buffalo	171.835	161.749	-5,9
Small ruminants	**56.265.750**	**52.363.410**	**-6,9**
Sheep	44.687.888	42.060.470	-5,9
Goat	11.577.862	10.302.940	-11,0

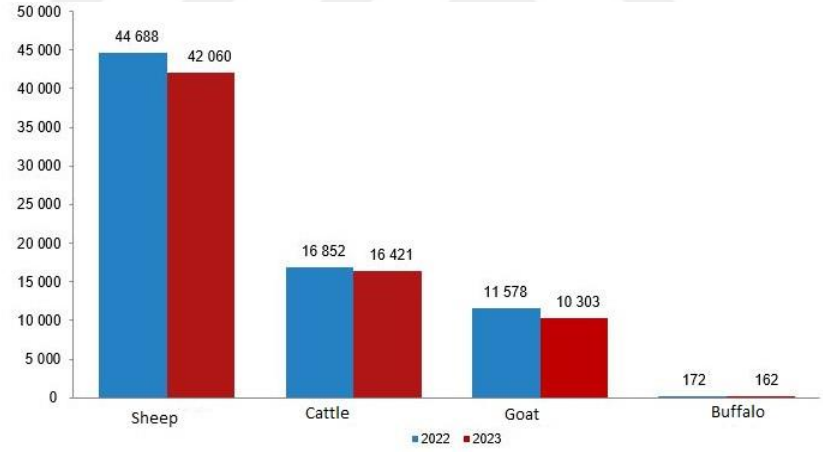

Figure 1 3. Number of ruminants in Türkiye (TÜİK, 2023).

1.1. Production and economic contribution of sheep

Domestic sheep differ from other species from their wild progenitors in their shape, color, and production of milk. The majority of the domesticated sheep breeds produce wool.

Many different breeds of sheep have been developed to suit climatic conditions as well as human demands for food and clothing. Fine-wool sheep breeds are usually raised for wool, while varieties with medium- or long-wool or oily hair are typically raised for meat. Numerous crossbreeds had been bred to produce high-quality meat and wool.

Livestock farming has important functions in economic development because of its balanced and healthy diet (Aslan et al., 2001). Sheep breeding is an important part of worldwide animal

production. Sheep still play an important role across the world. Wool is also used in the production of clothes, which are necessary for human survival (Emsen et al., 2008). Sheep were originally domesticated primarily for their meat. However, there was a notable development in the specialization of enterprises towards the production of 'secondary' products such as wool. This specialization occurred by the fifth millennium BP in South-West Asia and by the fourth millennium BP in Europe (Ryder,1983; Sherratt et al., 1981; Helmer et al., 2007).

Milk and its products are essential foods in human nutrition. Dairy sheep breeding constitutes an important part of the national economy, especially in the Mediterranean and Middle Eastern countries (Yerlikaya and Karagözlü., 2008). Sheep's milk, with its unique taste and smell and its casein content, is suitable for making yogurt. It's also preferred for butter production due to its high-fat content (Yerlikaya and Karagözlü, 2008).

Various animal food sources provide the nutrients humans need. One of the four main food groups recommended for ensuring sufficient intake of life nutrients is milk and dairy products (Yücecan, 2008). Based on FAO 2022 data, there were 2.4 billion small livestock. The total raw milk production is 887 million tons, of which 2.32% is goat milk and 1.20% is sheep milk (FAO, IFAD, UNICEF, WFP, and WHO, 2021; FAO, 2022b). According to (TÜİK, 2022), raw milk production was 92.3% of cow's milk, 4.9% sheep's milk, 2.5% goat's milk, and 0.2% buffalo's milk in Türkiye (Table 1.3 and Figure 1.4).

Table 1.3. Raw milk production in Türkiye (Ton) (TÜİK, 2022)

Year	Cow	Buffalo	Sheep	Goat	Total
2020	21.749.342	63.767	1.101.065	589.617	23.503.790
2021	21.370.116	63.643	1.143.762	622.785	23.200.306
2022	19.912.135	43.589	1.067.342	540.426	21.563.492

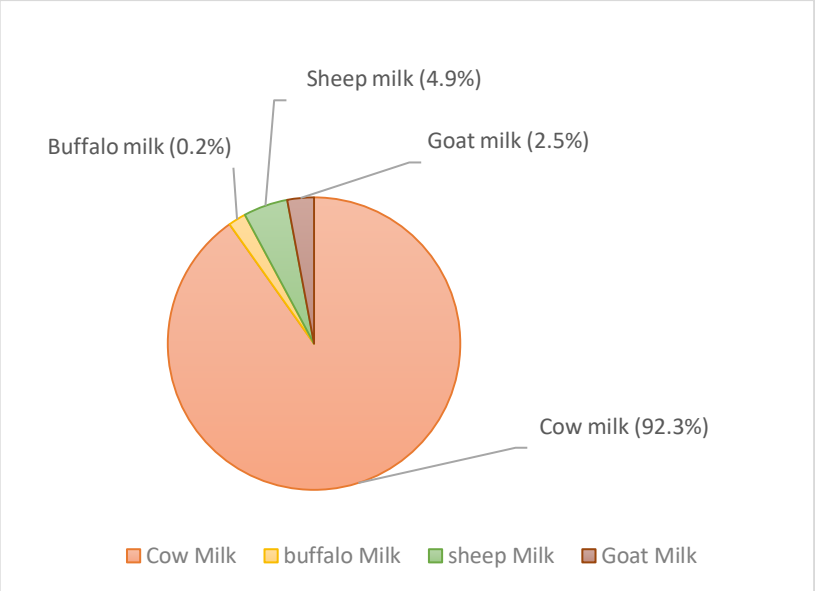

Figure 1.4. Distribution of raw milk production by species in Türkiye (%)

Sheep Meat: Meat and carcass attributes, such as quality of the meat and weight of carcass, are significant phenotypic characteristics that hold great value for human consumption. Meat quality is one of the most essential characteristics of meat and requires further attention in the meat industry. Meat quality is determined by fatty acid content, which can affect illnesses such as obesity, diabetes, neurological disorders, and cancer (Ntambi et al., 2004). Furthermore, fatty acid content affects the hardness and color of meat (Webb, E., and O'neill, H.A., 2008). Meat production accounts for the largest share of the gains from sheep farming. For the production of meat in sheep farming, it is necessary to increase the fertility and growth performance of lamb for meat production (Özcan et al., 2002). Breeders apply programs to increase the productive capacity of animals by changing their genetic composition; however, the duration (generation range) in animals like sheep can be as long as 4.5 years. To continue improving livestock, it is necessary to keep up with genetic improvement methods, study the genetic composition of these animals, and select the best of them by studying the genes that affect growth and production characteristics, comparing the genetic composition of the ÇET (Cukurova Meat Sheep) and Awassi sheep with global breeds, and identifying genetic mutations and linking them to their appearance using sequenced PCR (Polymerase Chain Reaction). Sequencing, overall, helps to study the required genes, and it is more experimental to determine the genetic composition of each animal, to read the nitrogen base sequence in the animal's DNA, and to detect mutations (Alain et al., 2002; Liu and Cordess., 2004). Sheep farming has traditionally played an important role in Turkey's livestock industry. People consume cheese and yogurt made from lamb and sheep's milk. Sheep breeding differs from other animals in that it converts low-quality grasses into milk and meat.

In 2021, the total red meat production in Türkiye was 1.952.038 tons and increased to 2.191.625 tons in 2022 (TÜİK, TÜİKSAT, 2023) (Table 1.4).

Table 1.4. Red meat production and rate of change by years in Türkiye

Animal	2021 (Ton)	2022 (Ton)	Change (%)
Cattle	1.460.719	1.572.747	7.7
Sheep	385.933	489.354	26.8
Goat	94.555	115.938	22.6
Buffalo	10.831	13.586	25.4
Total	1.952.038	2.191.625	12.3

In 2022, red meat production comprised 71.8% cattle, 22.3% sheep, 5.3% goat, and 0.6% buffalo meat (Figure 1.5).

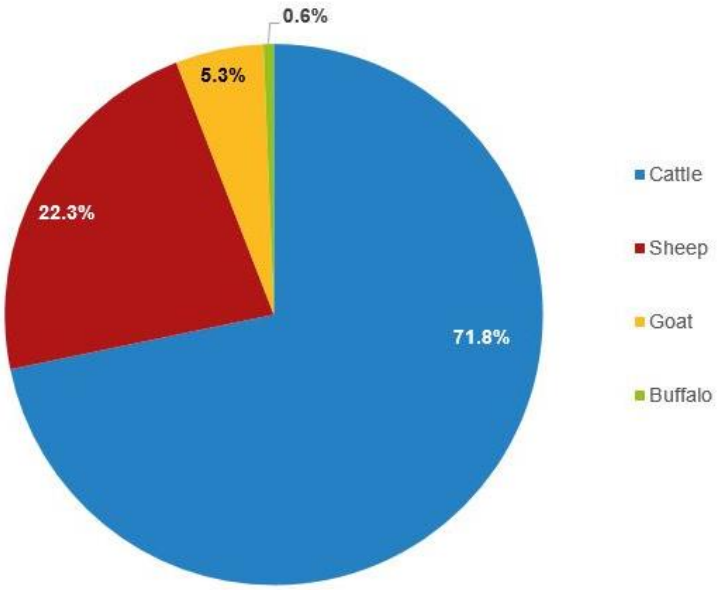

Figure 1.5. Red meat production in Türkiye in 2022 and its distribution by species (TÜİK, 2023).

1.2. Production of sheep in Türkiye

The climate, geographical, and economic conditions of the region to be cultivated are key factors determining the direction of farming. Along with all these factors, the farmer's objective are important requirements for milk or meat production. Farmers who want to increase their income by producing either meat or milk must carefully select the most suitable breed. When transferred to a place other than where they were bred, races that did not dramatically reduce income and yield were described as having good adaptations to the environment (Aydin et al., 2024).

There are 33 different species of sheep in Turkey. (Sheep and Goat Breeders' Associations of Türkiye, 2021). The sheeps in Türkiye are mostly fat-tailed, they grow up before the winter season. The fat stored in sheep's tails is a useful raw material during periods of poor nutrition and winter (Yilmaz et al., 2012; 2013).

1.2.1. Awassi Sheep

In Türkiye, the Awassi breed (Figure 1.6) is considered to be a dairy breed. The Awassi strain is well-known for yielding most milk following the East Friesian. Awassi is also has desirable traits in terms of resistance to nutritional variations, disease and parasite resistance, and tolerance to various temperatures, in addition to milk production and development abilities (Gürsoy et al., 1992, 1993). There are numerous varieties of Awassi sheep. Some of these varieties include Naiemi species in Kuwait, Iraq, and Syria, Shefali and Herrik in Iraq. In dairy farms, the udders of Awassi sheep are extremely developed, and teats are regularly placed (Alkass and Juma, 2005). The Awassi are bred in southern Turkey, Iraq, Israel, Jordan, and Syria. Awassi sheep are highly adapted to conditions of poor consumption and higher temperatures (Said et al., 1999).

Awassi meat has an intense taste, which is most caused by the fat's fatty acid content. Australians crossed Merino sheep with Awassi to obtain the required aroma for lambs sent to Arab countries (Gürsoy, 2005). In the Middle Eastern regions, the Awassi is the most common fat-tailed sheep.

Figure 1.6. Individuals of Awassi breed (Türkiye Evcil Hayvan Genetic Kaynakları, 2009)

1.2.2. Çukurova Meat Sheep (ÇET)

A crossbreeding experiment was conducted at Çukurova University to create a new sheep breed that is resilient to the climates of southeast Anatolia and possesses the traits of Awassi and Chios (Sakız) sheep. This resulted in the creation of a new crossbreed known as Çukurova Meat Sheep (Figure 1.7) (Özcan, 1989; Güney, 1990; Güney et al., 1997). They used four different types of sheep (37.5% Ile de France (İF), 37.5% Rambouillet (R), 12.5% Awassi (İ), and 12.5% Chios (S)), as shown in (Figure 1.8.).

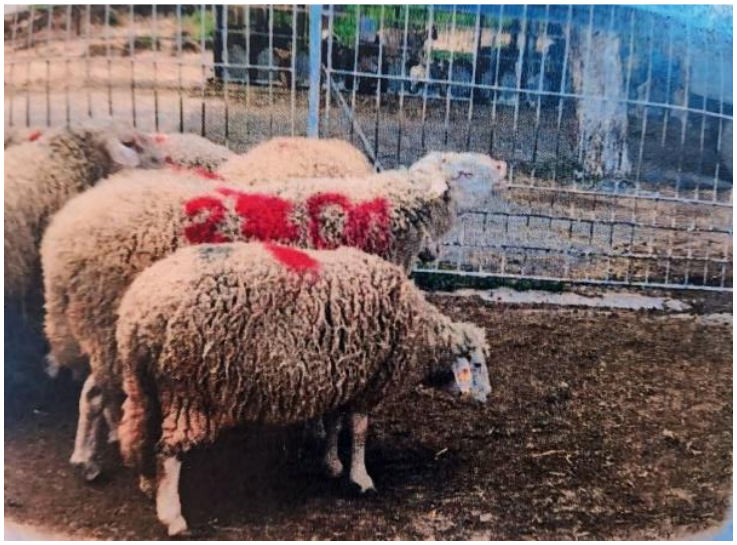

Figure 1.7. Individuals of ÇET breed

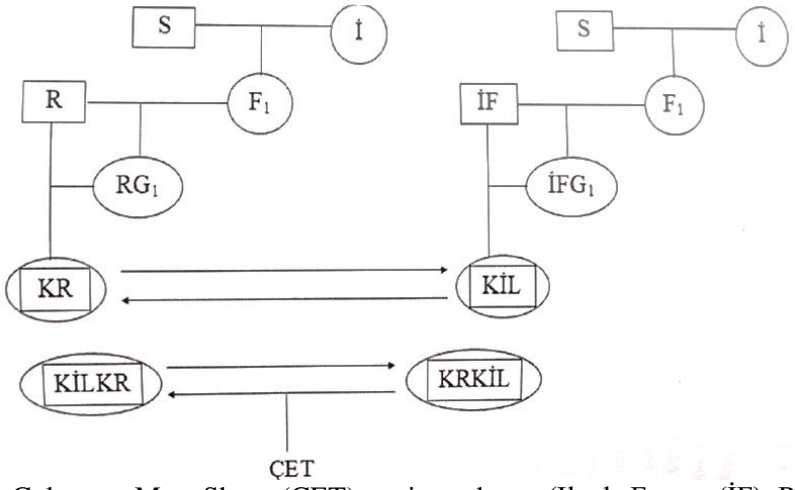

Figure 1.8. Çukurova Meat Sheep (ÇET) mating scheme (Ile de France (İF), Rambouillet (R), Awassi (İ), and Chios (S)) (Alsalihi, 2020)

1.3. Sheep breeding

Breeding and selection are the two primary tools in a breeder's hand that improve their herd or flock in the intended direction. For an animal breeder, selection involves choosing the best animals to produce following generations that collect more of the desired genes and genotypes of sheep (Vandana et al., 2018). Increasing animal productivity due to traditional breeding processes takes a long time.

The primary aim of animal breeding is to improve the genetics of individual animals in addition to those of future generations of the population. Effective selection depends on the identification of animals with superior hereditary traits (Johansson and Rendel, 1968). Animals are either kept or discarded for breeding purposes, depending on their phenotype for a specific trait. The progress of selection depends on how closely connected the genotype is to the phenotype. Individual phenotypic characteristics, like color and horn status, are used to determine breeding value. Evaluating large quantities or phenotype characteristics improves selection success. As a result, selection based on individuality is useful but may not always be completely correct (Tomar, 1996; Marleen et al., 2014).

1.4. Candidate genes affecting sheep productivity

1.4.1. Growth-related genes

The growth characteristics of sheep are considered to be significant economic traits. Advances in molecular genetics and genomics have greatly facilitated the discovery and understanding of genes associated with economic traits. The *MSTN* gene is related to muscle contraction and growth (Broad et al., 2000; Sahu et al., 2017). This gene is responsible for the production of growth hormone (GH), a regulatory protein in the body that has been identified as playing a significant role in the modulation of weight gain following the period of weaning in mammalian species. This particular genetic factor has been noted to exert an influence on the rate

and extent of weight gain experienced by individuals during the post-weaning phase, highlighting the intricate interplay between genetic determinants and physiological processes in the context of growth and development (Moradian et al., 2013).

Candidate genes (Table 1.5) such as *GRM1*, *MBD5*, *UBR2*, *RPL7*, and *SMC2* have been meticulously pinpointed and recognized at the genomic level for their potential correlation with post-weaning weight gain (Zhang et al., 2013). Myocyte enhancer binding factor 2B (*MEF2B*) and Thyrotropin-releasing hormone-degrading enzyme (*TRHDE*) are genes reportedly affecting body weight and growth in sheep (Zhang et al., 2016). The tumor protein p53 (*TP53*) gene, which plays an important role in DNA-binding transcription factor activity and apoptosis, has been related to the size of the body (Kominakis et al., 2017).

While growth is primarily controlled by the pituitary gland, there exist genes that influence the development of this gland. One illustration of such genes includes *LHX3* and *LHX4*, which play roles in body growth and reproductive functions in sheep (Park et al., 2013).

Table 1.5. Some candidate genes related with body weight and growth characteristics (Gebreselassie et al., 2019)

Genes	Traits	Sheep Breeds
LHX3	GT	Hu, KKHK, LYKHK, TK
LHX4	GT	Hu, KKHK, LYKHK, TK
MSTN	GT	MK, T, M
GH	GT	Makui sheep
GRM1	PWG	Sunit, Alman M., Dorper
MBD5	PWG	Sunit, Alman M., Dorper
UBR	PWG	Sunit, Alman M., Dorper
SMC2	PWG	Sunit, Alman M., Dorper
RPL7	PWG	Sunit, Alman M., Dorper
TP53	BS	Frizarta sheep

1.4.2. Meat quality and carcass related genes

Meat quality and the weight of the carcass are important meat characteristics. Meat quality is one of the factors that shows its effectiveness. Furthermore, fatty acid content influences the firmness and color of the meat. The A113G variation of the *LEP* gene was associated with total body fat weight (Barzehkar et al., 2009). The myostatin gene was determined to be associated with meat yield (Hickford et al., 2010). The genes *DGAT1* and *UCP1* have been related to the depth of subcutaneous fat in the carcasses (Mohammadi et al., 2013; Yang et al., 2014). CAST genes have

been identified to affect the quality and fatty acid content (Aali et al., 2016). The candidate genes that are associated with meat quality and carcass are summarized in Table 1.6.

Table 1.6. Some candidate genes are associated with meat quality and carcass (Gebreselassie et al., 2019)

Genes	Traits	Sheep breeds
LEP	CW	Curly crossbred lambs
	TBFW	Zel sheep
	WW	Zandi sheep
MSTN	MY	Romney
UCP1	SCFD	NZ, Romney, Suffolk
DGAT1	BFTW, SCFD	Lori-Bakhtiari, Zel

1.4.3. Milk yield and quality-related genes

Milk is the most essential animal product for humans. Sheep's milk yield includes protein, fat, casein, and lactose percentages. Environmental conditions and genetic differences mostly control the production potential of milk characteristics, genetically through many different genes. All of the genes mentioned in the below table are directly or indirectly related to characteristics such as lactation duration, lactation milk yield, milk yield, fat, and protein ratio in milk. The milk and milk quality-related genes are summarized in Table 1.7.

Table 1.7. Some candidate genes associated with milk yield and quality (Gebreselassie et al., 2019)

Genes	Traits	Sheep breeds
POU1F1	MY	Turkish Sheep
PALMD	MY	Italian Altamurana
RFP145	MY	Italian Altamurana
LALBA	MY	Spanish Churra
GH1	MY	Serrada Estrela
CSN2	MY, FR, PR	Polon Merinos

1.4.4. Fertility related genes

Fertility characteristics, including litter size, ovulation rate, age at initial lambing, total number of lambs born, stillbirth rates, and age at initial puberty, are essential factors that impact the economic dimensions of sheep farming. Especially the rate of ovulation and the high number of offspring born characteristics are high in economic value. All of the genes mentioned in the below table are related to fertility characteristics such as ovulation rate, follicle formation and

development, reproductive potential, and oocyte development. Fertility-related genes are summarized in Table 1.8.

Table 1.8. Some candidate genes are associated with fertility (Gebreselassie et al., 2019)

Gene	**Traits**	**Sheep Breeds**
BMPRIB	ORS	Lacaune sheep
GDF9	OD	Booroola
PRLR	RP	Turkish sheep
FecL	ORS	Lacaune
TMEM154	ILV	Rambouillet, Polypay, Columbia
CCNB2	OD	ÇMYK, Alman M, ABD
SLC8A3	OD	ÇMYK, Alman M, ABD

1.5. LIM homeobox transcription factor 4 (*LHX4*) gene function

The *LHX4* gene, belongs to the LIM (Lin11/lsl1/Mec3) homeobox gene family, is essential for developing the pituitary gland and neurological system. *LHX4* is a transcription factor that is involved in the regulation of stages of cells and plays a crucial role in pituitary development (Mehta et al. 2008). *LHX4* dysregulation has been linked to chromosomal translocations in several leukemia types (Yamaguchi et al., 2003). Mutations in the *LHX4* gene are related to CPHD (Combined Pituitary Hormone Deficiency) disorders in human and animal models affecting the growth hormone and anterior pituitary hormones (Li et al., 2008). This disruption results in delayed puberty and underdeveloped anterior pituitaries (Dattani 2004, Castinetti et al. 2008a). A mutation in LHX4 was found in a CPHD causing deficiencies in GH, TSH, and ACTH (Machinis et al., 2001). LHX4 gene mutations have been identified as causing CPHD in humans (Castinetti et al. 2008b; Pfaeffle et al. 2008).

A preliminary study reported a G>C mutation in the LHX4 gene, resulting in aberrant proteins unable to bind to a putative binding site within the POU1F1 promoter due to reduced DNA binding ability (Machinis and Amselem, 2005). POU1F1, GH, and PRL are known to play critical roles in multiple regulatory functions including milk production, growth, reproduction, endocrine functions, immunology, and illness (Wu and Xu, 2000; Cui et al., 2006; Vaclavicek et al., 2006; Lan et al., 2007). The significance of the LHX4 gene has been underscored through gene knockout studies in animals; mice with specific LHX4 mutations exhibited high mortality rates soon after birth (Li et al., 1994). Two single nucleotide polymorphisms in exon 3 altered the amino acid sequence of the LHX4 protein. A substitution in the LIM domain (R84C) reduced LHX4 protein activity (Pfaeffle et al., 2008). The mutation T99F in LHX4 resulted in amino acid sequence changes and impaired transcriptional activity in the POU1F1 promoter, thereby affecting PRL and GH promoter

transactivation (Castinetti et al., 2008b). Recent investigations highlighted LHX4's role in regulating PROP1, POU1F1, ACTH, GH, and PRL expression in the LHX3-LHX4-PROP1-POU1F1 pathway (Mullen et al., 2007; Mehta et al., 2008). Furthermore, a study identified a silent mutation in exon 6 of the LHX4 gene associated with body weight and length in Chinese cattle (Ren et al., 2010). No polymorphisms have been reported in other exons of the LHX4 gene. Further exploration of the LHX4 gene in livestock species is imperative.

In this thesis study, it was aimed to determine the polymorphism of the *LHX4* gene in two sheep breeds (Awassi and ÇET) in the Çukurova University Faculty of Agriculture Research and Application Farm Livestock Unit.

2. PRELIMINARY WORK

Li et al. (2008) used PCR-SSCP to analyze revealed polymorphic patterns in exon 6 of the goat LHX4 locus across eight different populations. They identified three distinct polymorphic patterns, known as the AA pattern consisting of two bands, the AG pattern with four bands, and the GG pattern displaying two bands. To investigate the genetic diversity within exon 6 of the goat LHX4 gene across various populations, ten amplified PCR fragments representing distinct patterns were subjected to bidirectional sequencing. Subsequently, a subset of these sequences was submitted to the Gen Bank. Comparisons were made between nucleotide sequences of the bovine LHX4 gene and other sequences, showing a new mutation. The sequences showed the G>A mutation at the 1053rd nucleotide caused a missense mutation at the LHX4 locus: Val>Ile at position 280. Compared to cashmere (IMWC and SBWC) and meat utility breeds (Boer and XH), dairy breeds (XS, LS, and GZ) have higher frequencies of allele A, the implication suggests allele A may be linked to milk performance. Therefore, the caprine LHX4 gene might have positive effects on dairy traits. In conclusion, with the novel missense mutation (1053G>A) the spectrum of genetic variation in the caprine LHX4 gene is expanded, aiding in association analysis and genetic marker evaluation in goat breeding, genetics, and CPHD detection.

Liu et al. (2011) identified polymorphisms within exons 1, 2, and 3 of the LHX4 gene in a cohort of 820 Chinese cattle, investigating their potential association with growth traits. Polymorphisms were detected using PCR-SSCP and DNA sequencing, revealing five synonymous mutations. The genotype MM and haplotype M predominated across all four cattle breeds studied. Genotype frequencies of LHX4 in these populations were consistent with Hardy-Weinberg equilibrium, yet varied significantly among the populations examined.

Ren et al. (2010) investigated the polymorphism at the LHX4-*Hae*III locus in a study involving Chinese cattle breeds. They were identified three genotypes GG, GA, and AA by using PCR-RFLP. The genotype GG frequencies ranged from 0.6620 to 0.9789 across the studied populations, all of which adhered to Hardy–Weinberg equilibrium. Significant variations in genotypic frequencies were observed among different cattle breeds. Genetic diversity analysis indicated that JX cattle exhibited intermediate diversity, whereas three other Chinese cattle breeds displayed lower genetic diversity. Notably, individuals of the Nanyang (NY) breed with genotype GA showed higher body weight and greater body length at 18 and 24 months compared to those with genotype GG.

Ren et al. (2014) investigated LHX4 polymorphisms associated with growth traits in beef cattle breeds. Seven SNPs were identified within coding and noncoding regions of the bovine LHX4 gene using pooled DNA sample sequencing and PCR-single strand conformation polymorphism (PCR-SSCP) techniques. Linkage disequilibrium was assessed in four major cattle breeds in China, revealing complete linkage disequilibrium between SNPs 2–5 and 7–8. Analysis of 13 SNPs

identified 31 haplotypes across the four Chinese cattle breeds, with 20 genotypes exclusive to Nanyang cattle. Statistical analysis did not find significant associations among the twenty combined genotypes. However, SNPs 1–5 and 6 were associated with body weight of Nanyang cattle at 18 and 24 months of age, indicating that LHX4 polymorphisms influence growth traits at specific developmental stages and may serve as markers for selective breeding and management in cattle.

Alkhammas et al. (2023) examined 232 ewes between the ages of 3 and 4 were chosen (109 ewes that had twins and 123 ewes that produced single progeny). Genomic DNA collected from sheep blood was isolated, subjected to genotyping, and subsequently sequenced to validate the presence of variations within LHX4 (specifically exon 1, spanning 207 bp). Genotyping identified three genotypes: AA, AG, and GG in 207 bp. A novel mutation, 160 A>G, was found in exon 1 through sequence analysis. Statistically 160 A>G SNP showed significant association with phenotypic traits. Ewes with AA genotypes showed elevated levels of certain hormones like estrogen, progesterone, follicle-stimulating hormones/luteinizing hormones, and growth hormone while having reduced levels of prolactin. Compared to AG and GG genotypes in twin-pregnant ewes with single-pregnant ewes in the fourth month.

Zhao et al. (2017) conducted a study involving four sheep breeds differing in fecundity levels, focusing on the analysis of unique indel variants within the LHX3 and LHX4 genes and their impact on growth parameters. They identified a single 29 bp indel within the sheep LHX3 gene, resulting in three distinct genotypes. Notably, the frequency of the II (insertion/insertion) genotype and the I allele showed a consistent increase with enhanced reproductive traits. Significant disparities were observed in both genotypic and allelic frequencies between the low-fecundity breed (TS) and high-fecundity breeds (HS, STHS, and LFTS). Association studies revealed significant differences in chest breadth and body length between female TS and STHS, as well as male STHS. This 29-bp indel expanded the spectrum of genetic variations in the sheep LHX3 gene, offering a valuable foundation for employing marker-assisted selection (MAS) in sheep breeding and genetics.

Yan et al. (2018) investigated a novel insertion/deletion (indel) within the LHX4 gene in 1149 Shaanbei white cashmere goats to explore its association with litter size. They identified a novel 12 bp indel located in the first intron, resulting in three genotypes with allelic frequencies of I (0.593) and D (0.407) in Shaanbei white cashmere goats. Notably, genotype distributions differed significantly between mothers of single-lamb and multi-lamb groups within this breed, suggesting a potential impact of the 12 bp indel on litter size. Association analysis indicated a significant correlation between the 12 bp indel and litter size in the studied goat population. Individuals with genotypes DD and ID exhibited larger litter sizes compared to those with genotype II. These findings contribute to the understanding of the functional genomics of goats and propose this locus as a potential genetic marker for selective breeding in goats.

3. MATERIAL AND METHOD

3.1. Animal materials

All experimental animals in this study were carried out in the Çukurova University Faculty of Agriculture Research of Small Livestock Unit on a total sample of 99 heads of sheep (48 Awassi and 51 Çukurova Meat sheep (ÇET) breeds. The genetic analyses were carried out in the Animal Biotechnology and Genetic Engineering Laboratory of the Department of Animal Science.

3.2. Collecting of blood samples

Genomic DNA samples were isolated from 99 sheep of two different sheep breeds, Cukurova Meat sheep (n=51 ÇET), and the Awassi sheep breed (n=48). For genomic DNA isolation, 10 ml of blood was obtained from the jugular vein (*Vena jugularis*) to the EDTA tube (Figure 3.1), then blood samples were placed in an ice box and transported to the laboratory. All blood samples were stored at -20 ºC until use.

Figure 3.1. Collecting of blood samples from animals

3.3. Genomic DNA extraction

The genomic DNA's were isolated from blood samples according to Miller et al (1988) using salt precipitation method (Figure 3.2). The experimental steps are given below:

Figure 3.2. Genomic DNA isolation from blood samples

1. Frozen blood samples were thawed at room temperature for 30 min.
2. 500 µl of each blood sample was taken and placed in 1.5 ml microcentrifuge tubes.
3. 1000 µl Erythrocyte Lysis Buffer Solution (Table 3.1) was added to the samples mixed by vortex for a short time and left at room temperature for 10 minutes.
4. The samples were centrifuged at 5000 rpm for 10 min.
5. The liquid part that was collected at the top of the microcentrifuge tubes (supernatant) was carefully removed. By observing the color of the pellets (cell part), the procedure was repeated (at most 3 times) with Erythrocyte Lysis Buffer Solution until the pellets turned white.
6. 1000 µl Physiological Buffer Solution (Table 3.1) was added to the pellets and mixed for a short time.
7. The samples were centrifuged at 5000 rpm for 7.5 min, and at the end of the centrifugation, the liquid was carefully removed again.
8. 400 µl Lysis TE Buffer Solution (Table 3.1) was added to the pellets and the pellets were ensured to dissolve thoroughly.
9. 100 µl SDS (Sodium Dodecyl Sulfate, 20% w/v) solution and 5 µl proteinase K (10 mg/ml) were added to the dissolved pellets, mixed gently, and kept in a water bath at 56 ºC for 1.5 hours. During incubation, the mixture was carefully mixed every 15 min.
10. The samples were removed from the water bath and then 250 µl of 6M NaCl Solution (Table 3.1) was added to the samples. The mixtures were mixed well by vortexing for 15 minutes.
11. The samples were centrifuged at 12000 rpm for 10 min, and the supernatant liquid containing DNA molecules was transferred to a new microcentrifuge tube.
12. After adding two volumes of 99.9% cold ethanol, the samples were mixed gently until the DNA strands clumped.

13. The samples were centrifuged at 10000 rpm for 7 minutes to ensure that the clumped DNA strands settled at the bottom of the tubes.
14. After centrifugation, the ethanol was removed and 1000 µl of 70% v/v ethanol was added to the DNA pellets. The tubes were mixed gently 4-5 times.
15. After centrifugation, the alcohol was removed, and DNA pellets were air-dried for 20 min.
16. 100 µl of TE Buffer Solution was added to DNA pellets, and the samples were kept overnight at 4°C to dissolve the DNA.
17. The purity of DNA was determined using a NanoDrop spectrophotometer (ND 1000) according to the OD_{260}/OD_{280} ratio. DNA samples with an OD_{260}/OD_{280} ratio of around 1.8 were used in PCR studies.

Table 3.1. Molarity/amount and contents of buffer solutions used in DNA isolation

Buffer Solutions	**Contents**	**Molarity/Amount**
Erythrocyte Lysis Buffer Solution	Sucrose	0.32 M
	EDTA	10 mM
	$MgCl_2$	5 mM
Physiological Buffer Solution	NaCl	75 mM
	EDTA	25 mM
Lysis TE Buffer Solution	Tris-HCl	500 mM
	EDTA	20 mM
	NaCl	10 mM
TE Buffer Solution	Tris	10 mM
	EDTA	1 mM
6M NaCl Solution	NaCl	35.064 mM
	bdH_2O	Complete to 100 ml

3.4. Agarose gel electrophoresis of DNA molecules

In this study, 0.6% agarose gel used for electrophoresis of genomic DNA and %1 agarose gel used for electrophoresis of PCR amplicons.

For this purpose, the appropriate amount of agarose was calculated, weighed, and added to the appropriate volume of 1X TBE solution (Tris-Borate-EDTA) (Table 3.2) (Figure 3.3) and boiled in a microwave oven for 45 seconds. The gel mixture was allowed to cool at room temperature to 48°C and then homogenized by adding 1 µl of Ethidium bromide (EtBr, 10 mg/ml). After the mixture was poured into the prepared gel tray, a gel comb was placed and the gel was allowed to polymerize for 30 min (Figure 3.4). At the end of the time, the gel was taken to the electrophoresis tank, and the comb was removed from the gel. 1X TBE buffer was added to the electrophoresis tank just above the gel. DNA samples (DNA: Loading dye (5:1 v/v)) were added to the wells with the help of a

micropipette and after anode-anode and cathode-cathode connections were made with the power supply of the electrophoresis device, the samples were electrophoresed at 80 V, 40 mA for 120 min. At the end of the time, the electrical connection was turned off, the gel was removed from the electrophoresis tank and the DNA samples were observed at UV 260 nm wavelength with the help of a transilluminator. When necessary, a 100 bp DNA ladder (Solis BioDyne, 07-11-00050) was used.

Table 3.2. Components of the 5X TBE buffer

Agent	**Amount**	**Final concentration**
Tris base (pH 8.0)	54 gr	0.445 M
Boric acid	27.5 gr	0.445 M
EDTA (0.5 M, pH: 8.0)	20 mL	0.01 M
Distilled water	Up to 1000 mL	
[1]TBE solution was autoclaved at 121 °C for 15 min		
[2]For 1X concentration, 200 mL of 5X TBE buffer was added to 800 mL of distilled water		

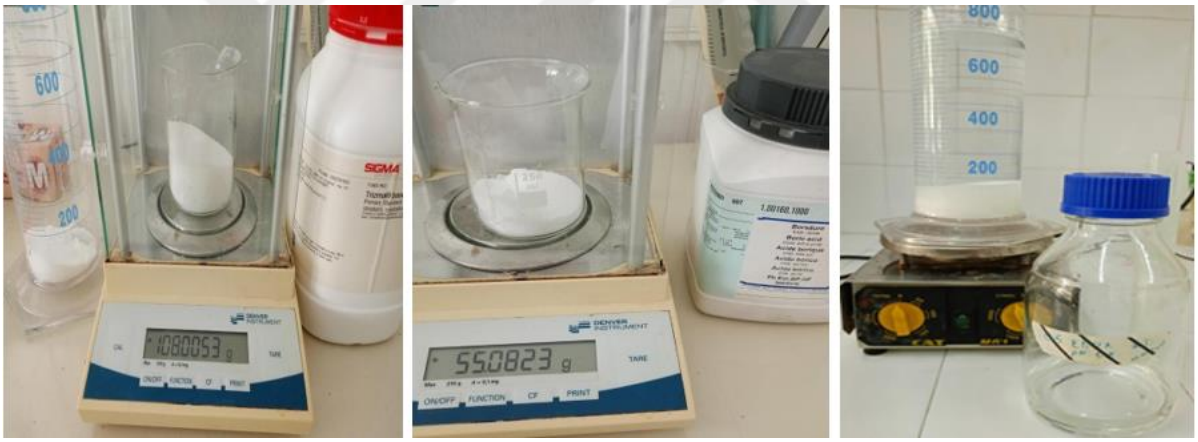

Figure 3.3. Preparation of 5X TBE buffer

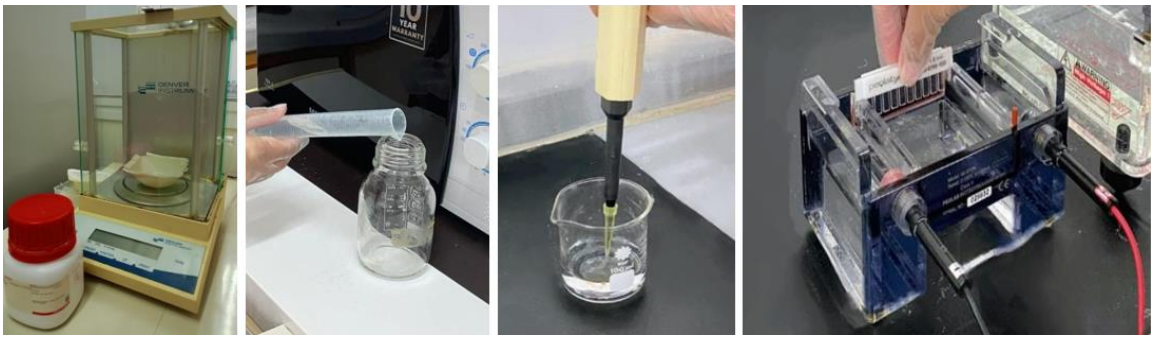
Figure 3.4. Preparation of agarose gel

3.5. Primer design, PCR amplification

According to Zhao et al. (2017), two sets of primers were used to amplify the *LHX4* of sheep growth and reproduction-related genes (Table 3.3). For stock primer solutions, all lyophilized primers were dissolved in de-ionized distilled water as suggested by the supplier to obtain the last concentration of 100 pmol/µl (100 µM) (Figure 3.5). The primers were centrifuged for 1-2 minutes before adding the distilled water, and all components came to the lower of the tube. The tube did not shake after adding the water, kept overnight in the refrigerator at +4°C. The next day, primers were vortexed and stored in a freezer at -20 °C. To obtain 20 pmol/µl (100 µM) for daily use, 20 µl of primer and 80 µl of water were added and mixed gently. Appropriate annealing temperatures for LHX4-P2 and LHX4-P3 primer sets were determined by gradient PCR reactions (Figure 3.6). The PCR cycling protocol is given in Table 3.4. Each amplicon was visualized with electrophoresis on 1% agarose gels in 1X TBE buffer.

Table 3.3. PCR primer sequences of the sheep *LHX4* gene

Primer	Sequences (5′→3′)	Gene size (bp)	**Reference**
LHX4-P2	F: CCCCAACACCTCAAACCTCTT	294	Zhao et al. (2017)
	R: TCCGATGTAGGCATGGGAAC		
LHX4-P3	F: GGAGGAAAGTGTCAACTGGG	294	
	R: AGCAATAGACGGCGGAGC		

F: Forward primer; R: Reverse primer

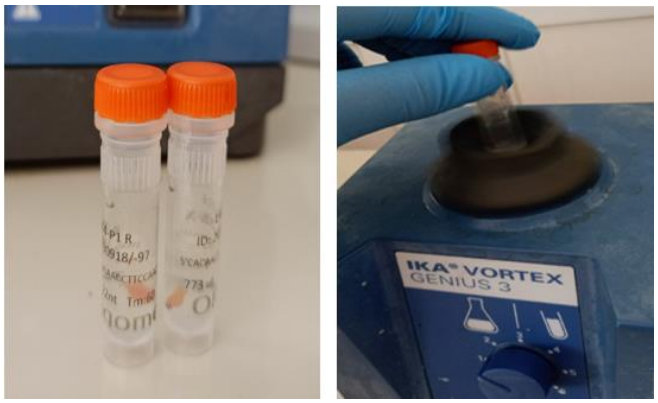

Figure 3.5. Preparation of 100 pmol stock solution of primer sets.

Figure 3.6. Gradient PCR protocols for LHX4-p2 and LHX4-p3 primers

Table 3.4. PCR cycling protocol for amplification of *LHX4* gene

Step	**Temperature (°C)**	**Time**	**Cycles**
Initial denaturation	95	5 min	1
Denaturation	95	1 min	30
Annealing	60.7 (For LHX4-P2) 61.6 (For LHX4-P3)	1 min	
Elongation	72	90 sec	
Final elongation	72	10 min	1
Storage	4	∞	1

3.6. Polymerase chain reaction (PCR)

The PCR Master Mix (Solis BioDyne, 04-11-00125) (Figure 3.7) was used during the synthesis of the genes. To determine the optimal amplification conditions for the study, different concentrations of primers and DNA templates were used in the PCR reaction, as well as different temperatures, time periods, and cycles, depending on the primers used. For PCR applications of the genes, the optimal conditions for amplification were used. Bio-Rad: PCR Thermal Cycler (Figure 3.8) was used in all PCR studies. Amplification reaction conditions for the *LHX4* gene are shown in Table 3.5.

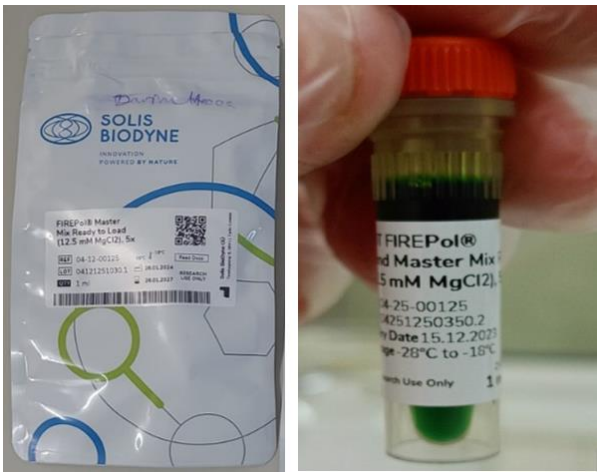

Figure 3.6. PCR Master Mix used in PCR reactions

Figure 3.7. PCR Thermal Cycler (Bio-Rad) used in amplification of the LHX4 gene

All PCR components were mixed in a separate tube (1.5 mL) at the correct concentrations, except the template DNA, and liquated 19 µl into each PCR tube (0.2 mL). 1 µl of Genomic DNA from each animal was then added to the PCR tubes, bringing the volume to 20 µl (Figure 3.9).

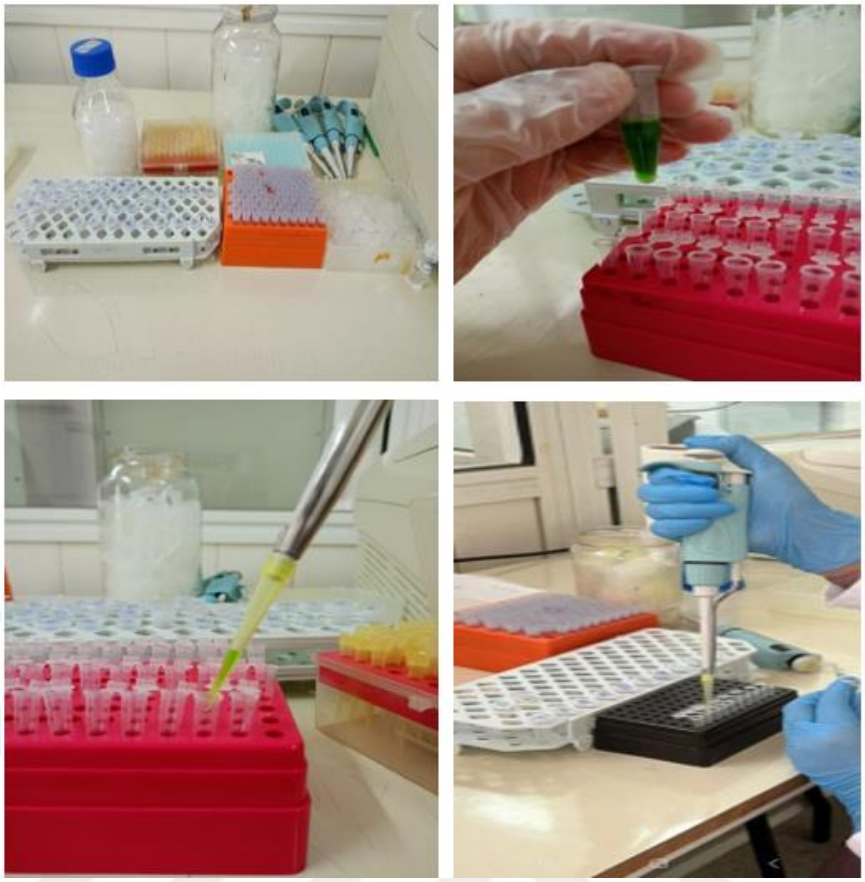

Figure 3.8. Preparation of PCR reaction components

Table 3.5. The PCR reaction constituents are required to amplify the LHX4-P2 and LHX4-P3 genes.

Reaction Component	**LHX4-P2**		**LHX4-P3**	
	Amount	**Final Concentration**	**Amount**	**Final Concentration**
Master mix (5X)	4 µl	1X	4 µl	1X
F-Primer (10 µM)	0.5 µl	0.25 µM	0.5 µl	0.25 µM
R-Primer (10 µM)	0.5 µl	0.25 µM	0.5 µl	0.25 µM
DNA template	1 µl	170 ng	1 µl	220 ng
Steril bdH_2O	14 µl	-	14 µl	-
Total volume	20 µl	-	20 µl	-

After completing the PCR cycling, the amplified DNA samples were separated electrophoretically using an agarose gel.

3.7. Statistical analysis

In this study, one-way analysis of variance (One-Way ANOVA) was used to determine the effect of LHX4-P2 and LHX4-P3 gene polymorphisms on various growth traits in Awassi and ÇET sheep. Analyses were performed using Minitab 19 software. POPGENE version 1.32 was used to calculate the Hardy-Weinberg test ($P \leq 0.05$) for estimated values of allele and genotype frequencies. The following statistical model was employed in the analysis of variance using One-Way ANOVA:

$$Yij=\mu+\alpha i+\epsilon ij$$

- Yij: Phenotypic value
- μ: Overall means
- αi: Genotypes effect
- ϵij: Random errors

4. RESULTS AND DISCUSSIONS

4.1. Isolation of genomic DNA

Genomic DNAs were isolated from the blood samples of 48 Awassi and 51 ÇET sheep included in the study (Figure 4.1). All isolated genomic DNAs were checked for quality on a 0.5% agarose gel before being used in PCR studies (Figure 4.2).

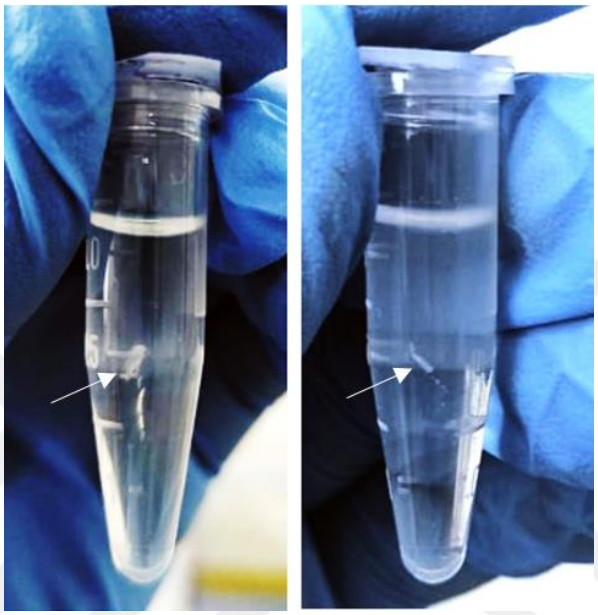

Figure 4.1. Precipitated genomic DNA in ethanol

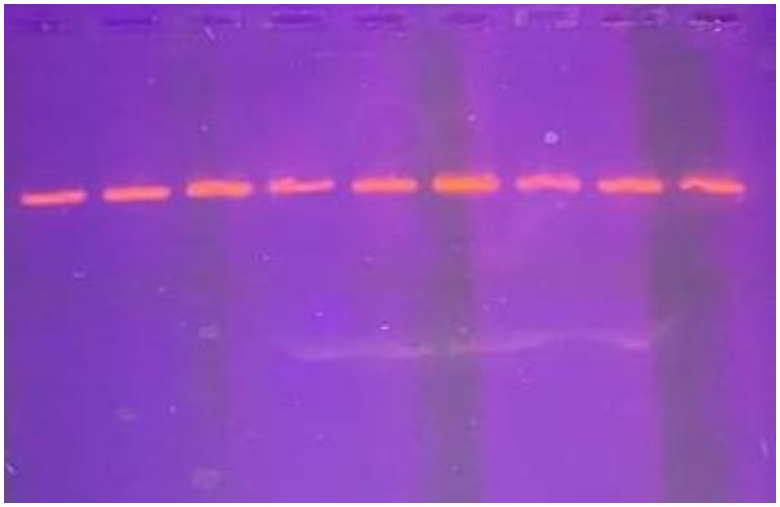

Figure 4.2. Agarose gel electrophoresis of genomic DNA samples

4.2. LHX4-P2 results

This study comprised the analysis of genomic DNA samples obtained from 66 sheep**.** Isolated the DNA using salt precipitation method. After PCR amplification, the PCR product was visualized under UV light using an agarose gel electrophoresis, and PCR was used to produce sequencing data that identified a 12 bp insertion or deletion (indel) in the *LHX4* gene. Two genotypes

(II and DD) were found. Genotype II had a 294 bp band, while genotype DD had a 282 bp band. LHX4-P2 PCR results revealed 19 DD genotypes and 29 II genotypes in the Awassi breed, while no heterozygous genotypes were found (Figure 4.3). These results revealed that the frequencies of II and DD genotypes in the LHX4-P2 gene region in the Awassi breed were 0.58 and 0.42, respectively.

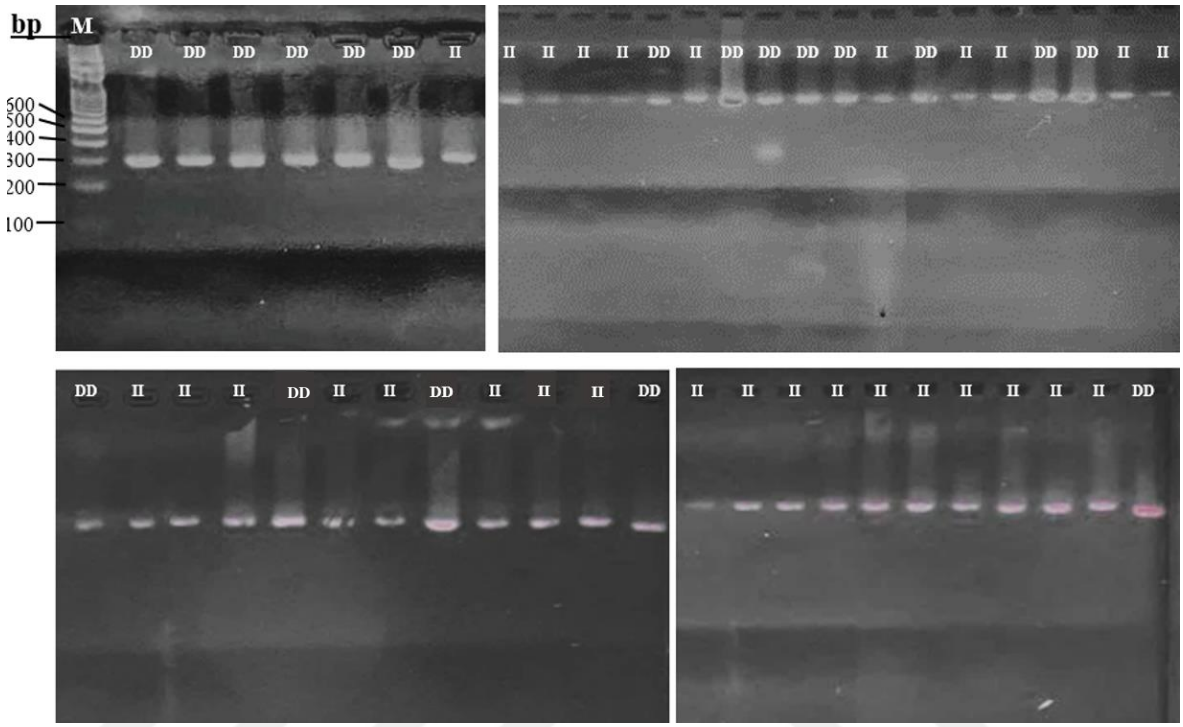

Figure 4.3. Electrophoresis pattern in Awassi sheep LHX4-P2 gene on 1% agarose gel (II: 294 bp, DD: 282 bp, M: 100 bp DNA ladder)

On the other hand, 11 animals with the II genotype and 7 animals with the DD genotype were found in the LHX4-P2 gene region in the ÇET sheep breed, while no heterozygous genotypes were found (Figure 4.4). These results revealed that the frequencies of II and DD genotypes in the ÇET breed were 0.60 and 0.40, respectively.

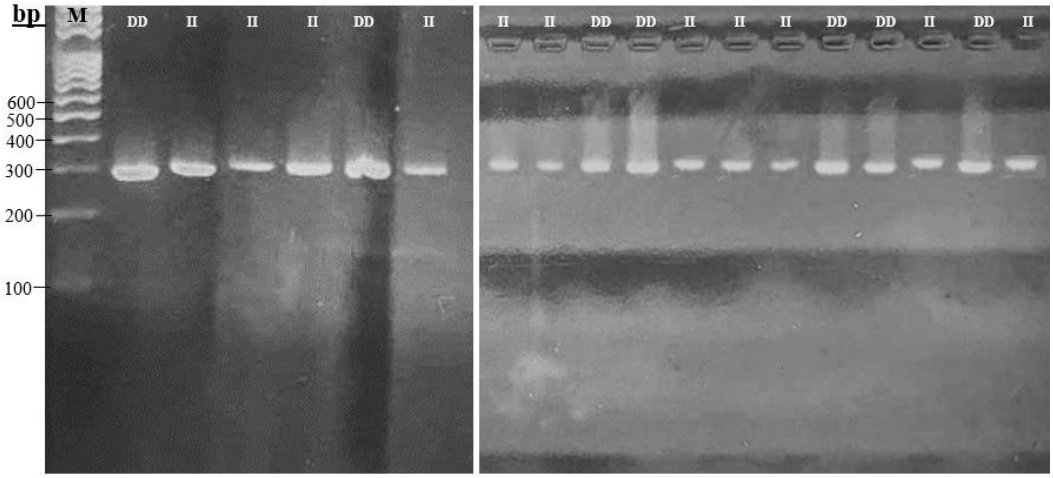

Figure 4.4. Electrophoresis pattern in ÇET sheep LHX4-P2 gene on 1% agarose gel (II=294 bp, DD=282 bp, M: 100 bp DNA ladder)

4.3. LHX4-P3 results

LHX4-P3 PCR results revealed 30 DD genotypes and 17 II genotypes in the Awassi breed, while no heterozygous genotypes were found (Figure 4.5). These results revealed that the frequencies of II and DD genotypes in the LHX4-P3 gene region in the Awassi breed were 0.38 and 0.62, respectively (Figure 4.5).

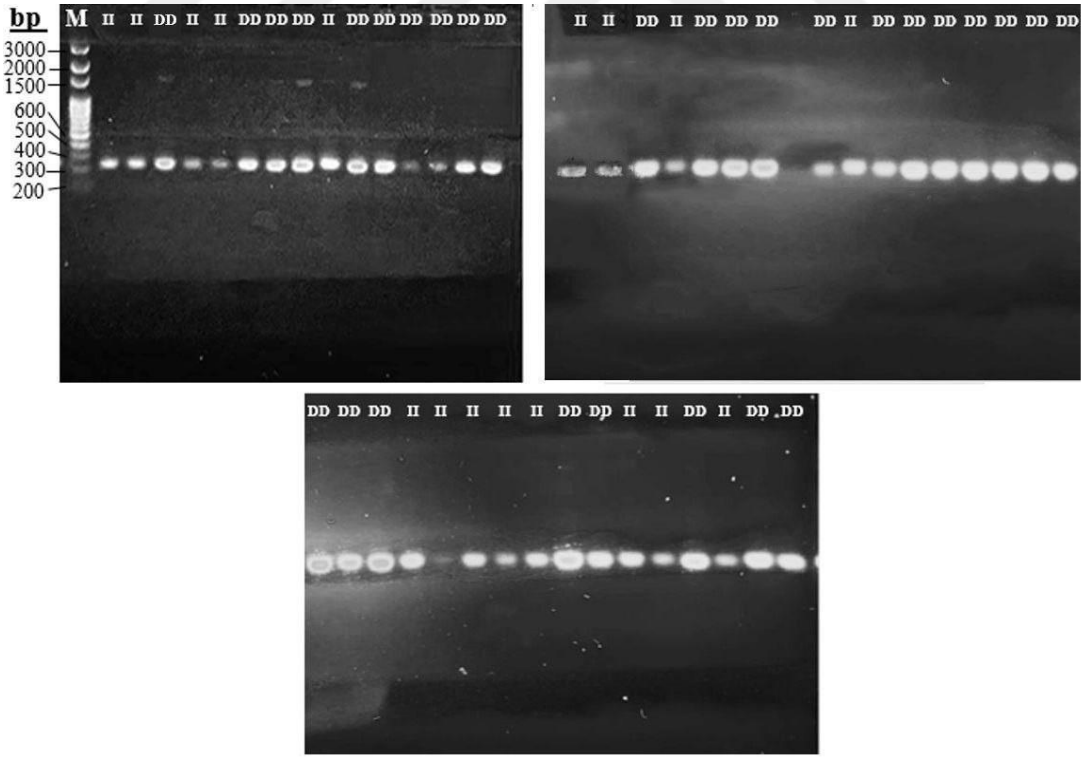

Figure 4.5. Electrophoresis pattern in Awassi sheep LHX4-P3 gene on 1% agarose gel (II: 294 bp, DD: 282 bp, M: 100 bp DNA ladder)

On the other hand, 13 animals with the II genotype and 38 animals with the DD genotype were found in the LHX4-P3 gene region in the ÇET sheep breed, while no heterozygous genotypes were found

(Figure 4.6). These results revealed that the frequencies of II and DD genotypes in the ÇET breed were 0.30 and 0.70, respectively.

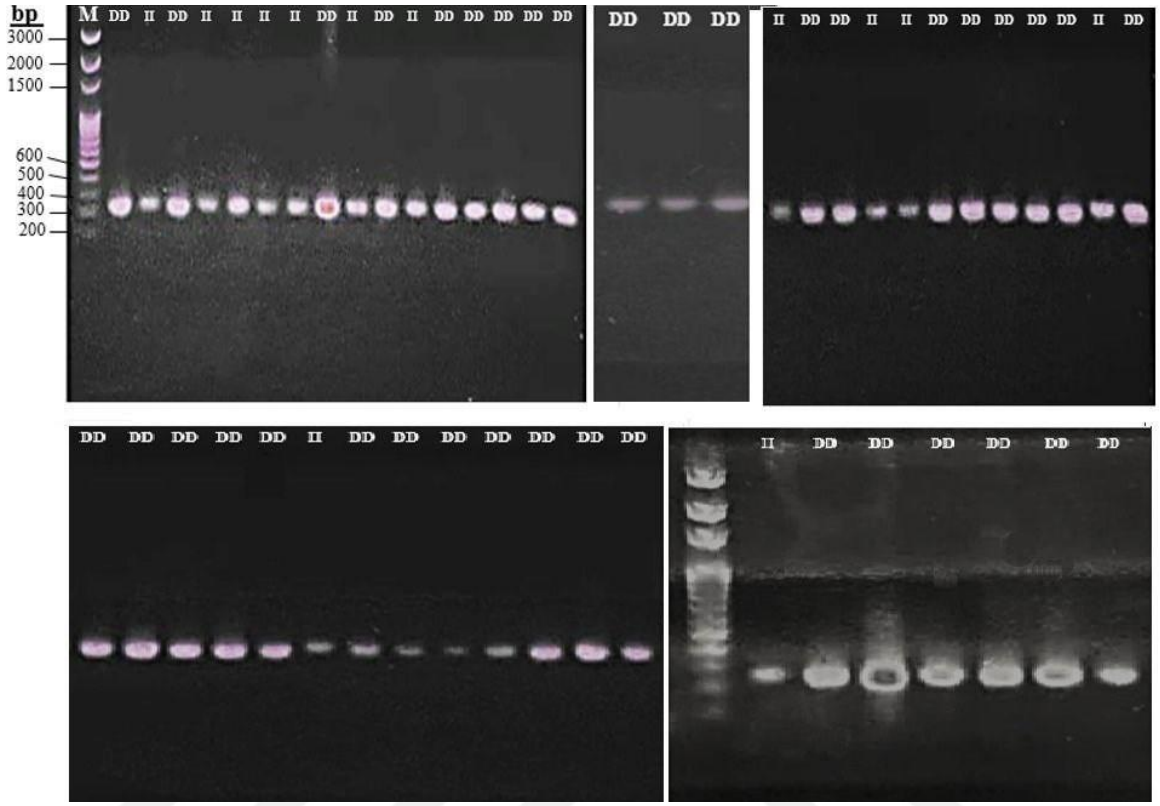

Figure 4.6. Electrophoresis pattern in ÇET sheep LHX4-P3 gene on 1% agarose gel (II=294 bp, DD=282 bp, M: 100 bp DNA ladder)

4.4. Genotype and allele frequencies

The results indicate that the LHX4-P2 genotype is significantly deviating from Hardy-Weinberg equilibrium in both the ÇET and Awassi sheep breeds ($p < 0.05$). In the case of the ÇET sheep, the observed genotype frequencies were 40% DD and 60% II, while the allele frequencies were 40% D and 60% I. Similarly, the genotypic frequencies observed in the Awassi sheep were 42% DD and 58% II. The allelic frequencies were 42% D and 58% I. On the other hand, the results of the study showed that the LHX4-P3 gene locus deviated from Hardy-Weinberg equilibrium in both ÇET and Awassi sheep breeds ($p<0.05$). In ÇET sheep, DD genotype frequency was found to be 70% and II genotype frequency was found to be 30%. Allele frequencies were 70% for D and 30% for I. In Awassi sheep, DD genotype frequency was 62% and II genotype frequency was 38% and allele frequencies were 62% for D and 38% for I. The absence of heterozygous genotypes in the population ensured that the genotypic and allelic frequencies were equal (Table 4.1).

Table 4.1. Genotype and allele frequencies of LFX4-P2 and LHX4-P3 gene polymorphism in ÇET and Awassi breeds

Locus	Breed	Genotype Frequencies		Allele Frequencies		χ^2	P Value
		DD	II	D	I		
LHX4-P2	ÇET	0.40	0,60	0.40	0,60	15	0.001*
	Awassi	0.42	0.58	0.42	0.58	43	0.001*
LHX4-P3	ÇET	0.70	0.30	0.70	0.30	47	0.001*
	Awassi	0.62	0.38	0.62	0.38	42	0.001*

χ^2, Chi-square test, *P<0.05

4.5. Association of LHX4-P2 and LHX4-P3 gene polymorphism with growth traits

The relationship between LHX4-P2 and LHX4-P3 gene polymorphisms and growth traits was investigated in Awassi and ÇET sheep. No significant relationship was found between the genotypes (DD and II) and the loci (LHX4-P2 and LHX4-P3) in both breeds (Table 4.2).

Table 4.2. Association of LFX4-P2 and LHX4-P3 gene polymorphism in ÇET and Awassi breeds

Breed	**Locus**	**Traits**	**Genotypes**		**P value**
			DD	II	
ÇET	LHX4-P2	Height at wither (cm)	70.17 ± 1.19	68.33 ± 1.18	0.313
		Height at rump (cm)	70.67 ± 1.74	68.89 ± 1.01	0.36
		Chest depth (cm)	32.33 ± 1.12	31.56 ± 0.65	0.529
		Body length (cm)	65.17 ± 2.34	64.22 ± 1.15	0.695
		Chest width (cm)	22.83 ± 1.08	22.11 ± 0.48	0.504
		Rump width (cm)	25.67 ± 1.61	24.67 ± 0.83	0.555
		Chest girth (cm)	93.83 ± 3.65	91.11 ± 1.81	0.473
		Body weight (kg)	55.38 ± 1.59	48.38 ± 2.52	0.445
	LHX4-P3	Height at wither (cm)	69.15 ± 0.59	70.73 ± 1.11	0.255
		Height at rump (cm)	69.08 ± 0.59	70.71 ± 1.08	0.257
		Chest depth (cm)	32.29 ± 0.52	32.45 ± 1.06	0.877
		Body length (cm)	63.33 ± 0.68	65.36 ± 1.69	0.27
		Chest width (cm)	21.58 ± 0.37	23.09 ± 0.70	0.057
		Rump width (cm)	25.16 ± 0.43	26.36 ± 0.95	0.225
		Chest girth (cm)	92.96 ± 1.23	94.64 ± 2.57	0.568
		Body weight (kg)	56.230 ± 1.85	60.57 ± 4.03	0.31
Awassi	LHX4-P2	Height at wither (cm)	68.52 ± 0.98	70.03 ± 0.71	0.127
		Height at rump (cm)	68.64 ± 1.07	69.64 ± 0.68	0.296
		Chest depth (cm)	30.12 ± 0.86	31.57 ± 0.53	0.103

		Body length (cm)	63.04 ± 1.23	63.46 ± 0.88	0.769
		Chest width (cm)	19.56 ± 0.49	20.18 ± 0.37	0.258
		Rump width (cm)	22.24 ± 1.01	23.07 ± 0.47	0.433
		Chest girth (cm)	86.00 ± 1.68	87.79 ± 1.05	0.319
		Body weight (kg)	50,13 ± 1.91	52,11 ± 1.57	0.54
	LHX4-P3	Height at wither (cm)	70.62 ± 0.63	70.80 ± 1.02	0.871
		Height at rump (cm)	70.15 ± 0.57	70.10 ± 0.81	0.956
		Chest depth (cm)	31.06 ± 0.73	32.17 ± 0.56	0.299
		Body length (cm)	62.40 ± 0.83	65.63 ± 1.59	0.054
		Chest width (cm)	19.42 ± 0.45	20.17 ± 0.48	0.29
		Rump width (cm)	22.25 ± 0.56	23.90 ± 0.81	0.092
		Chest girth (cm)	86.31 ± 1.13	88.73 ± 1.31	0.182
		Body weight (kg)	49.67 ± 1.36	52.99 ± 2.54	0.213

4.6. Discussion

The objective of the present study was to investigate the LHX4 gene polymorphism in Awassi, and ÇET sheep breeds and to analyze their genotypic and allele frequencies associated with growth traits.

The genotypes DD and II were identified. The II genotype was shown to be the predominant genotype in Awassi and ÇET sheep breeds for the LHX4-P2 locus, while the DD genotype exhibited the highest frequency in both sheep breeds for the LHX4-P3 locus. In contrast, the ID genotype was not observed in any of the investigated breeds. Statistical analysis showed that no significant relationship was found between the genotypes (DD and II) and the loci (LHX4-P2 and LHX4-P3) in both sheep breeds. Zhao et al. (2017) identified LHX3 and LHX4 genes in 4 sheep breeds (Hu sheep (HS), small-tail Han sheep (STHS), Lanzhou fat-tail sheep (LFTS), and Tong sheep (TS)) with four different primers (LHX3-P1, LHX3-P2, LHX3-P3, LHX3-P4, LHX4-P1, LHX4-P2, LHX4-P3, and LHX4-P4) and correlated the genotype frequencies with growth traits, they did not find any polymorphism in the LHX4 gene. However, in another study on sheep, Alkhammas and AL-Thuwaini (2023) associated the type of birth and genotypes (AA, AG and GG) caused by the A>G transformation at position 160 of the LHX4 gene sequence with reproductive hormones and prolactin levels in Awassi ewes, and they observed higher levels of estrogen, progesterone, follicle-stimulating hormones/luteinizing hormones and growth hormone and lower prolactin levels in ewes carrying AA genotype compared to AG and GG genotypes in the fourth month of twin-pregnant ewes compared to single-pregnant ewes. However, we could not find any other study in which the polymorphism of the LHX4 gene was associated with growth traits in sheep. However, in a study in goats, it was shown that a 12 bp indel mutation in the first intron of the LHX4 gene in Shaanbei white cashmere goats,

individuals with DD (deletion/deletion) and ID (insertion/deletion) genotypes were significantly superior to than individuals with II (insertion/insertion) genotypes for litter size (Yan et al., 2018). Another study in some Chinese goat breeds showed that statistical differences in genotypic frequencies due to G>A change in the exon 6 of the LHX4 gene, which causes GTT (Val)>ATT (Ile) conversion at position 280 of the amino acid sequence, were significantly associated with goat breeds (Li et al., 2008).

In a study conducted on cattle, Ren et al. (2010) associated the polymorphism in the LHX4 gene with the body weight and body length in Nanyang cattle. They demonstrated that the animals with genotype GA had greater body weight and longer bodies than those with genotype GG. On the other hand, Ren et al. (2014) showed that some SNPs found in the LHX4 gene were associated with body weight at 6, and 18 months of age in Nanyang cattle. In another study on cattle, 5 SNPs (G>A, C>T, C>T, G>A, T>C) in exons 1, 2, 3 of the LHX4 gene in the Nanyang breed were associated with growth traits, and it was shown that individuals with the MM genotype (GG-CC-CC-GG-TT) had a higher body weight at 18 months of age than individuals with the MN genotype (G/A-CC-CC-GG-TT) (Liu et al., 2011).

According to the literature review, this thesis represents the second study aimed at associating the indel mutation in the LHX4 gene with growth traits in sheep, and none of these studies have successfully established a correlation between the indel mutation and growth traits.

5. CONCLUSIONS

In conclusion, this study identified genotypes associated with a 12 bp indel mutation in the LHX4 gene using LHX4-P2 and LHX4-P3 primers in Awassi and ÇET sheep breeds. The relationships between the obtained genotypes (DD and II) and growth traits such as height at the wither (cm), height at the rump (cm), chest depth (cm), body length (cm), chest width (cm), rump width (cm), chest girth (cm), and body weight (kg) were investigated. However, no association was found between these genotypes and growth traits in either breed. The absence of this relationship suggests that genotyping the LHX4 gene using LHX4-P2 and LHX4-P3 primers will not contribute to breeding programs aimed at improving growth traits in these two sheep breeds.

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